American Journal of Pathology, Vol. 142, No. 3, March 1993

Demonstration of Extensive Chromatin Cleavage in Transplanted Morris Hepatoma 7777 Tissue: Apoptosis or Necrosis?

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Cell death may occur by either of two mechanisms: necrosis or apoptosis (programmed cell death). In this paper, we demonstrate extensive chromatin cleavage into oligonucleosome-length fragments (DNA ladder) in transplanted Morris hepatoma 7777 tissue, which is suggestive of the stimulation of an endogenous endonuclease activity previously found to be involved in the process of apoptosis. The existence of many apoptotic cells, which are morphologically characterized by condensed cytoplasm and basophilic nuclear fragments, were also seen in this tissue. In vivo and in vitro experiments were designed to further differentiate the morphological and biochemical features of necrosis and apoptosis in liver and hepatoma cells. Liver tissue undergoing ischemic necrosis showed a distinct DNA ladder pattern without demonstrating the morphology of apoptosis, indicating that chromatin cleavage into oligonucleosomal-length fragments is not confined to apoptotic cell death, at least in liver cells. In in vitro–cultured McA-RH7777 cells, however, DNA ladder pattern was detected only in cells showing characteristic morphology of apoptosis. From these two criteria (i.e., characteristic morphology and DNA ladder), it was strongly suggested that the apoptotic process is highly activated in the transplanted 7777 tissue. Based on the results obtained from in vitro experiments, it was suggested that tumor apoptosis may represent a residual attempt at autoregulation within the expanding tumor population and/or may result from mild cellular injuries such as hypoxia, nutrient deficiency, or other unknown noxious factor(s). We also showed evidence that apoptosis is inducible in hepatoma cells in vitro by a wide range of mild injuries or stimuli. (Am J Pathol 1993, 142:935–946)

It has been widely accepted that there is a marked discrepancy between the rate of enlargement of many malignant tumors and the rate of proliferation of their component cells. This was attributed to spontaneous cell death.[1] There are two major distinct modes of death in eukaryotic cells: necrosis and apoptosis.[2,3] These two processes differ both morphologically and biochemically. Necrosis, which usually involves groups of contiguous cells, is an uncontrolled destructive phenomenon induced by a variety of non-physiological agents (i.e., ischemia, toxic chemicals, infectious organelles, etc.) that disturb energy-producing pathways and create an osmotic imbalance in the cell, leading to swelling of cytoplasm and irreversible failure of cellular structures, caused by lysosomal enzymes.[4–6] Apoptosis, on the other hand, is the term for programmed cell death, which plays an important role in controlled deletion of cells during metamorphosis, differentiation, hormone-dependent atrophy, and normal cell turnover and is regulated by signal transduction-coupled events.[2,3] Recent accumulating evidence has expanded the definition of apoptosis, and it is now generally accepted that apoptosis is not always physiological.[7,8] Many kinds of injurious agents are shown to be able to induce apoptosis in many kinds of cell lineages.[9–11] The mechanisms that permit cells to die in this stereotyped fashion are now the major concern in this field.

Apoptosis is characterized morphologically by cell shrinkage and hyperchromatic nuclear fragments[3] and biochemically by chromatin cleavage into nucleosomal oligomers.[12–14] In this paper, we

Supported by U. S. Public Health Service grants CA42965 and CA25098 awarded by the National Cancer Institute.

Accepted for publication August 26, 1992.

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demonstrate extensive chromatin cleavage into oligonucleosome-length DNA fragments in Morris hepatoma 7777 tissue transplanted in the thigh muscle of rats. Although it is generally accepted that this DNA fragmentation, known as a "DNA ladder", is a characteristic biochemical indicator for apoptosis,[12–14] the possibility that the DNA ladder might also be implicated in the process of hepatoma necrosis still has not been excluded. This possibility seems to be further strengthened by the following facts. Earlier work has shown that a Ca^{2+}-dependent endonuclease exists in nuclei of many types of cells including liver cells.[15,16] It is also well recognized that the process of coagulative necrosis, which is a common feature of death in injured liver cells, is closely associated with the accumulation of cytosolic Ca^{2+}, which may result in the activation of various Ca^{2+}-dependent enzymes including endonucleases.[4–6,17] Stimulation of endogenous endonuclease activity in liver cells is indeed shown in several types of cell injury.[18] So the question is now open as to whether the DNA fragmentation into nucleosomal oligomers observed in 7777 tissue is due to apoptosis or coagulative necrosis. The experiments described in this report were designed to answer these questions and to elucidate the possible mechanisms for cell death in 7777 hepatoma tissue.

Materials and Methods

Tumor Cells and Cell Culture

Transplantable Morris hepatoma 7777 cells were grown in the thigh muscle of Buffalo rats. The McA-RH7777 cell line, derived from the Morris hepatoma 7777, was obtained from the American Type Culture Collection (Rockville, MD) and grown in Dulbecco's modified Eagle's medium supplemented with 15% heat-inactivated fetal bovine serum and antibiotics (100 μg/ml of streptomycin and 100 units/ml of penicillin) at 37 C and 5% CO_2. Cells were seeded at 1×10^5/ml and cultured for 24 hours before treatment. The serum-free synthetic medium (SFM) used in the experiments of serum starvation was RPMI 1640 supplemented with 3×10^{-8} mol/L sodium selenite and other trace elements according to the procedure of Nakabayashi et al.[19] For experiments investigating the effect of calcium depletion on cell death in culture cells, a number of agents were tested under both calcium-sufficient and calcium-deficient conditions. For calcium-deficient conditions, cells were cultured in Ca^{2+}-free Dulbecco's modified Eagle's medium containing 15% dialyzed fetal bovine serum (GIBCO Laboratories, Grand Island, NY).

In Vivo *Induction of Liver Cell Necrosis*

Male Wistar rats weighing approximately 150 g (6 weeks) were used for the following experiments that induce liver cell necrosis *in vivo*. The experiments were performed according to the institution's guidelines for the care and use of laboratory animals in research.

Ischemic Necrosis

Under ether anesthesia, the peritoneal cavity was opened and blood vessels for the left lateral lobe of the liver were ligated. After closure of the peritoneal cavity, the rats were maintained in a cage with feeding *ad libitum*. Treated rats were killed (0.5, 1, 2, 4, 8, 12, 24, and 48 hours after the treatment), and liver samples were removed.

Acute Carbon Tetrachloride (CCl_4) Hepatotoxicity

Rats were injected intraperitoneally with CCl_4 (1.25 ml/kg of body weight), mixed with an equal volume of olive oil. Animals were killed at 6, 12, 24, 36, 48, 72, and 96 hours after the injection, and liver tissue samples were removed. Liver tissue samples for biochemical analysis were immediately frozen and stored at −80 C. Small slices were processed for light microscopy after fixation with 10% buffered formalin and embedding in paraffin. The degree of cellular injury was assessed histologically after hematoxylin and eosin (H&E) staining.

In Vitro *Induction of Cell Death in Hepatoma Cells*

Monolayer cells were treated with the following injurious agents at various dosages to induce cell death; Ca^{2+} ionophore A23187, ethanol, ricin lectin, cycloheximide, and ultraviolet (UV) exposure. The specific biological activity of A23187 (Sigma Chemical Co., St. Louis, MO) is to create Ca^{2+} channels and thereby to overcome the permeability barrier represented by the cellular membrane with the resultant influx and intracytoplasmic accumulation of Ca^{2+} that leads to cell death.[20] A23187 was dissolved in dimethylsulfoxide to make a 25-mmol/L stock solution and stored at −20 C. Ricin lectin (EY Laboratories, Inc., San Mateo, CA) and cycloheximide (Sigma) have inhibitory effects on protein synthesis. For UV exposure, cells were cultured in a tissue culture

dish of 10 cm diameter (Falcon 3003, Becton-Dickinson, Lincoln Park, NJ) with 9 ml of medium, and the monolayer culture cells were exposed to various doses of UV using the Stratalinker UV crosslinker (Stratagene, La Jolla, CA), which has a built-in detector to ensure consistent UV energy for each experiment. Irradiated cultures were subsequently incubated under standard conditions.

DNA Isolation and Agarose Gel Electrophoresis

DNA was isolated according to the method described by Sambrook et al[21] with minor modifications. In brief, tissue and cells were directly homogenized in DNA extraction buffer (10 mmol/L Tris-HCl, pH 8.0, 100 mmol/L ethylenediaminetetraacetic acid, 0.5% sodium dodecyl sulfate, 100 μg/ml RNAse A) and incubated for 1 hour at 37 C followed by incubation for 3 hours (for culture cells) or 16 hours (for tissue) at 50 C in the presence of 100 μg/ml of proteinase K. The DNA was then extracted with phenol/chloroform and precipitated in ethanol. Pellets were resuspended in $T_{10}E_1$ (10 mmol/L Tris-HCl, pH 8.0/1 mmol/L ethylenediaminetetraacetic acid), and the DNA concentration was determined from the absorbance at 260 nm. Each DNA sample (20 μg) was electrophoresed through a 1.6% agarose gel containing 1 μg/ml of ethidium bromide. DNA bands were visualized with a UV transilluminator and photographed.

Results

Morphological Signs of Cell Death by Apoptosis in Transplanted Morris Hepatoma 7777 Tissue

Scattered single cell death was frequently detected in the 7777 tissue. Under light microscopy, these dead cells showed the following histological features that are characteristic of apoptosis (Figure 1A): 1) acidophilic cytoplasm and nuclear fragments appearing as discrete basophilic masses and 2) the absence of inflammatory reaction. Several mitotic cells were also observed in the vicinity of apoptotic cells, indicating that stimulation of tumor growth and apoptosis were concurrent and that ischemia and/or nutritional deficiency are not the major cause for the scattered single cell death in the 7777 hepatoma tissue. Apoptotic and mitotic cells were scored by counting 10,000 cells in random fields of the hepatoma tissue. Apoptotic cells and mitotic cells accounted for 7.6% and 4.3% of the total cell number, respectively. Together with the frequent appearance of a cluster of apoptotic cells, the higher incidence of apoptotic cells than mitotic cells may suggest the accumulation of dead cells in tumor tissue, probably due to immaturity of drainage vessels and/or a lack of phagocytes. The tissue also contained regions showing cell death of contiguous tumor cells whose histological distribution is very similar to that of ischemic necrosis (Figure 1B). Although apoptosis was originally characterized as single cell death, the cells in these regions also showed fragmented and condensed nuclei, which are usually not observed in necrotic cells. Feulgen staining clearly demonstrated condensed chromatin in fragmented nuclei of these dead cells in 7777 tissue (Figure 1C). These findings strongly suggest that the apoptotic process is implicated in the cell death of both scattered and contiguous distribution in 7777 hepatoma tissue. Taking these contiguous cell deaths into account, cells undergoing apoptotic cell death were estimated to occupy around 20 to 30% of total cell number in the 7777 hepatoma tissue.

Demonstration of DNA Ladder in 7777 Tissue

Agarose gel electrophoresis revealed a characteristic pattern of DNA fragments in transplanted 7777 tissue. The DNA fragments consisted of multimers of 180 to 190 base pairs, consistent with internucleosomal cleavage of chromatin DNA by an endonuclease (Figure 1D, lane 2). DNA isolated from normal rat liver was detected only in the high-molecular-weight region (Figure 1D, lane 1).

DNA Fragmentation in Liver Tissue Induced By Ischemic Necrosis or CCl_4 Hepatotoxicity

After ligation of both the hepatic artery and the portal vein, the tissue of the ischemic lobe showed the histological pattern of coagulative necrosis (Figure 2A). Both cytoplasm and nuclei showed decreased stainability with hematoxylin and eosin. Fragmentation or condensation of the cytoplasm and nuclei were not observed. DNA fragmentation was not significant in liver tissue taken at 30 minutes and at 1 hour after ligation. The characteristic DNA ladder pattern was observed in the liver taken 2 hours after the ligation in which the tissue seemed to be at an early stage of ischemic necrosis. At later time points, the steps in the ladder became smaller due to progressive DNA hydrolysis. However, even at the latest

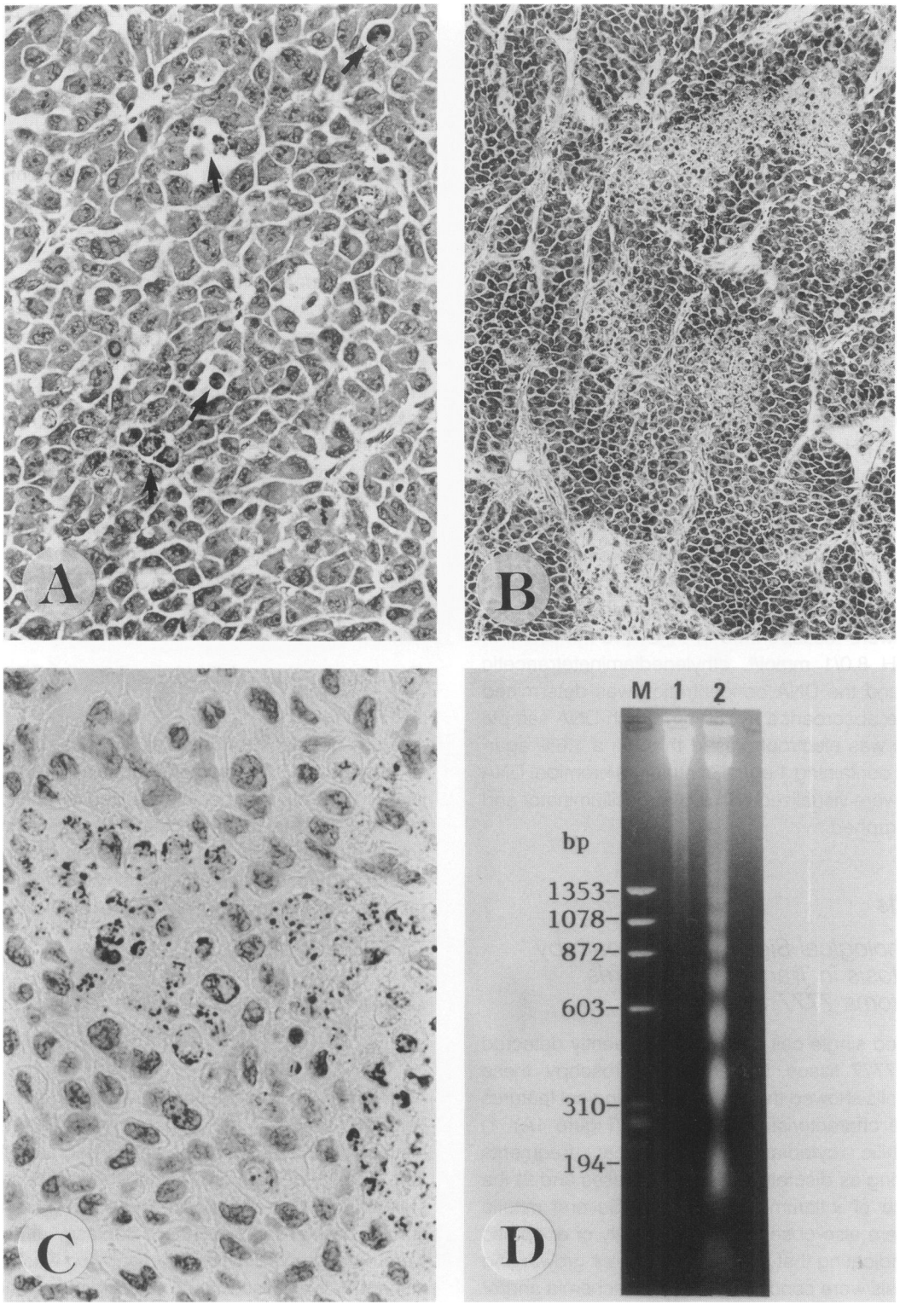

Figure 1. *Apoptotic features in transplanted Morris hepatoma 7777 tissue.* A: *Many scattered apoptotic cells (arrows) are present. These cells show acidophilic cytoplasm with condensed and fragmented nuclear materials. No inflammatory cell infiltration is seen. Several mitotic cells are observed in close vicinity to apoptotic cells (H&E, ×281).* B: *Regions showing cell death of contiguous tumor cells are frequently observed in which histological distribution is very similar to that of ischemic necrosis (H&E, ×70).* C: *Condensation and fragmentation of nuclear materials are clearly demonstrated in dead cells by Feulgen staining (Feulgen stain, ×562).* D: *Analysis of DNAs isolated from normal rat liver* (lane 1) *and transplanted Morris hepatoma 7777 tissue* (lane 2) *with 1.6% agarose gel electrophoresis. Note extensive DNA fragmentation of multi-nucleosomal units (DNA ladder) in* lane 2, *suggesting the process of apoptosis.* Lane M *contains DNA molecular weight markers.*

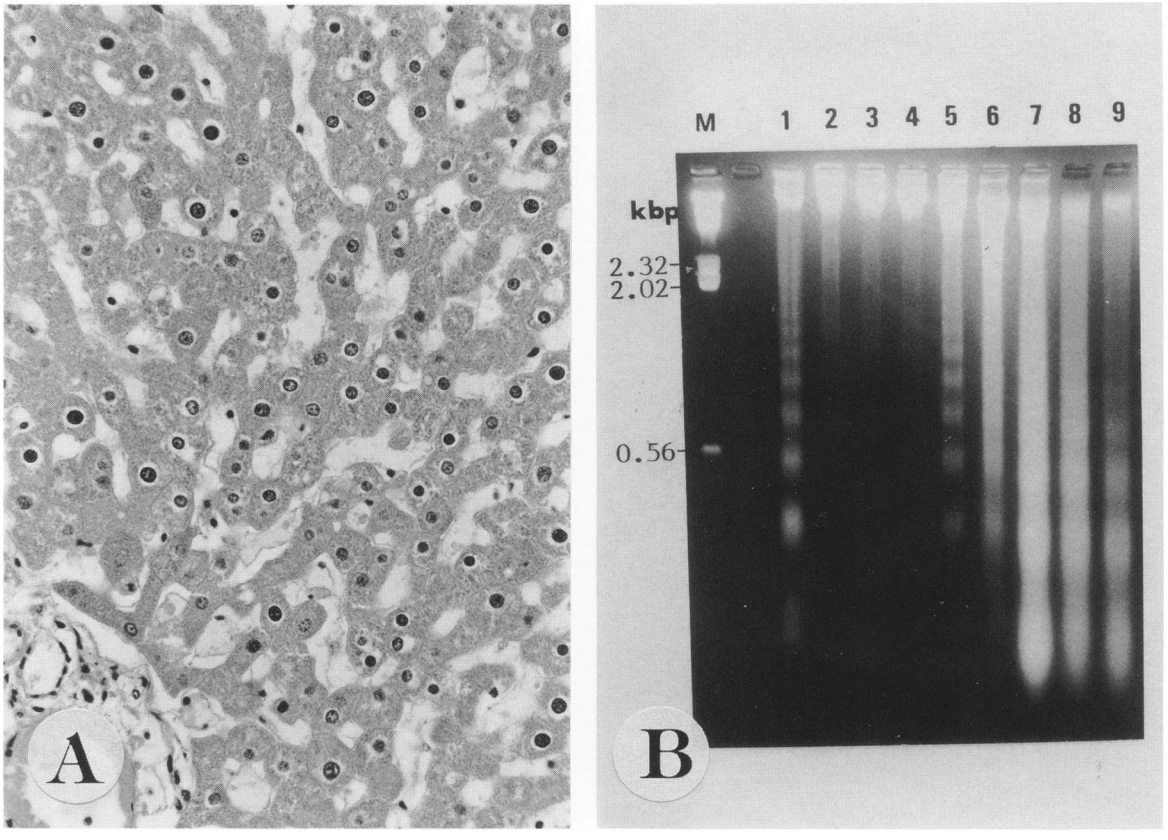

Figure 2. *Histology* **(A)** *and DNA fragmentation pattern* **(B)** *of the ischemic liver tissue.* **A:** *The liver tissue obtained 48 hours after the ligation of the blood vessels shows definite histology of coagulative necrosis, which is demonstrated by decreased stainability with (H&E) and by a decrease in the cellular size. Condensation or fragmentation of cytoplasm and nuclei, which are characteristic to apoptosis, are not observed (H&E, ×312).* **B:** *Electrophoretic analysis of DNAs isolated from 7777 hepatoma tissue* **(lane 1)**, *normal liver* **(lane 2)**, *and ischemic liver lobes that had been ligated for 30 minutes* **(lane 3)**, *1 hour* **(lane 4)**, *2 hours* **(lane 5)**, *4 hours* **(lane 6)**, *8 hours* **(lane 7)**, *12 hours* **(lane 8)**, *and 24 hours* **(lane 9)**.

time point (24 hours after ischemia), the ladder pattern was observed against a background of a smear (Figure 2B). The hepatotoxin CCl_4 causes an acute centrilobular necrosis (Figure 3A). Histological signs of cellular injury such as fatty degeneration and centrilobular necrosis were observed 12 to 48 hours after the single injection of CCl_4. The liver tissue taken 96 hours after the toxic injury showed marked improvement due to removal of necrotic debris and regeneration of remaining hepatocytes. The DNA isolated from the liver tissue having centrilobular necrosis showed a faint DNA ladder pattern (Figure 3B). The DNA ladder pattern observed in CCl_4-treated liver tissue was, however, very faint in spite of extensive cell death (1/4 to 1/3 of the parenchymal cells) of the coagulative necrosis type. Although both ischemic and CCl_4-intoxicated liver tissue showed the coagulative necrosis type of cell death, the DNA fragmentation pattern was very different. This indicates that the DNA fragmentation process differs in necrotic cells depending on the injury. Furthermore, our results clearly provided evidence that the DNA ladder, which has been widely accepted as a biochemical indicator for apoptosis in many cell lineages, can be produced in liver cells undergoing ischemic necrosis without demonstrating the characteristic nuclear morphology of apoptosis.

Because transplanted 7777 hepatoma tissue already contained many dead cells and showed the distinct DNA fragmentation of the ladder pattern, it was difficult to evaluate the effects of ischemia and injurious agents in the hepatoma tissue. In order to differentiate the morphological features and the DNA fragmentation pattern in necrotic and apoptotic cell death in hepatoma cells, the following *in vitro* experiments were carried out using Morris hepatoma 7777–derived cell line McA-RH7777.

Induction of Necrosis and Apoptosis in Cultured Hepatoma Cells

The McA-RH7777 cells were treated to various kinds of toxic injury at various dosages, and the dead cells were investigated to determine the morphological

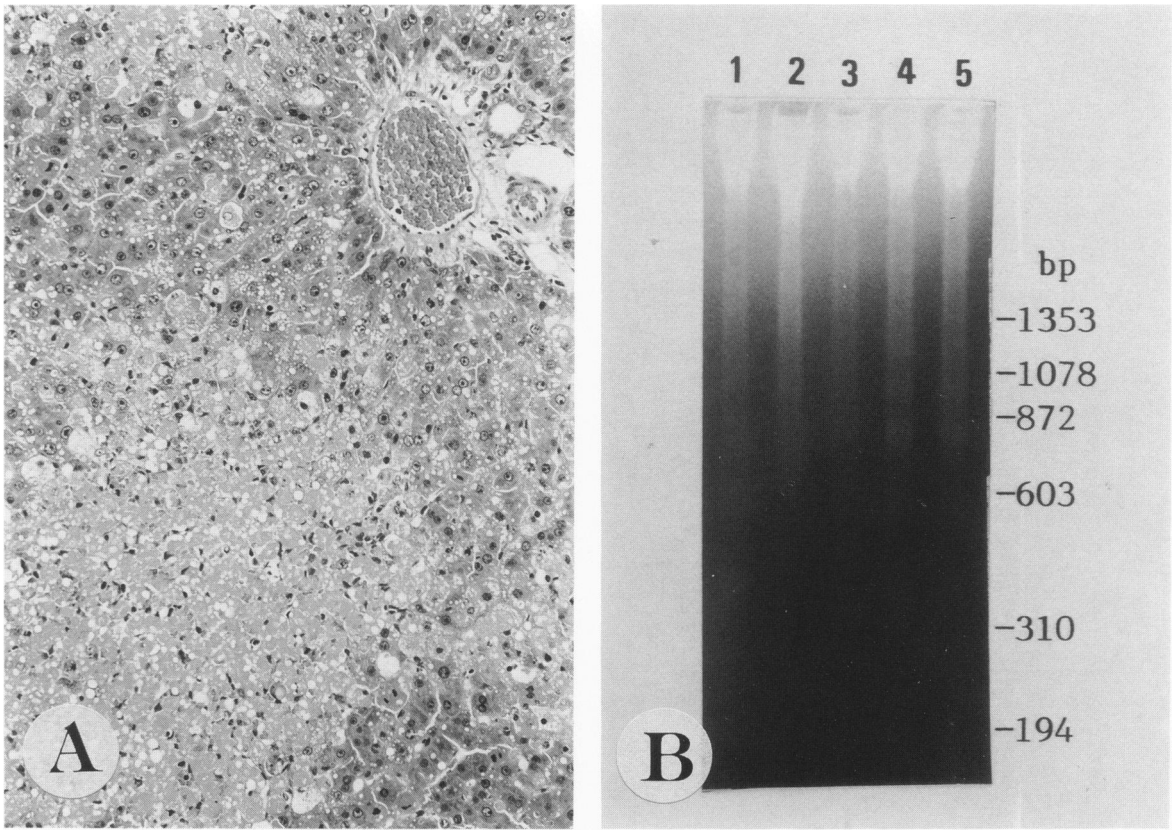

Figure 3. *Histology* **(A)** *and DNA fragmentation pattern* **(B)** *of the liver tissue after a single dose of CCl_4 (1.25 ml/kg of body weight).* **A:** *Histology of the liver tissue taken 24 hours after a single dose of CCl_4, showing centrilobular coagulative necrosis and fatty degeneration (H&E, ×156).* **B:** *Electrophoretic analysis of DNAs isolated from the liver tissue taken 6 hours* **(lane 1)**, *12 hours* **(lane 2)**, *24 hours* **(lane 3)**, *36 hours* **(lane 4)**, *and 48 hours* **(lane 5)** *after a single dose of CCl_4.*

changes and the pattern of DNA fragmentation. Apoptosis and necrosis were morphologically quite different. Apoptotic cells were easily identifiable on H&E-stained samples by cell shrinkage with condensed and fragmented nuclei. The criteria that governed the identification of necrotic cells included cytoplasmic swelling and loss of nuclear basophilia, eventually giving rise to karyolysis. Cells in the final stages of necrosis underwent cell lysis, in direct contrast to what takes place during apoptosis. Analysis of DNA from necrotic cells revealed no specific cleavage of the DNA, indicating random fragmentation of the DNA.

From these two criteria, hepatoma cells were shown to have two major pathways in the process of cell death in spite of a variety of methods used to initiate injurious action. One is direct cell death that was induced shortly after severe cellular damage. Another type is indirect cell death in which the cells die several hours after initiating injury. The latency period from initiating injury to cell death varies according to the severity and type of injury. Direct cell death showed findings characteristic of necrosis, and indirect cell death showed characteristic features of apoptosis. For example, direct cell death was induced in cultured McA-RH7777 cells by a high dosage of ethanol (more than 10%), UV exposure (more than 3×10^3 mJ/cm^2), or Ca^{2+} ionophore A23187 (more than 20 μmol/L). Figure 4 shows the morphology and the DNA fragmentation pattern in dead cells induced by Ca^{2+} ionophore A23187. When the McA-RH7777 cells were treated with A23187 at concentrations of more than 20 μmol/L, almost all cells died within a few hours. High doses of UV irradiation also induced necrotic cell death in the hepatoma cells: cells irradiated with 3×10^3 mJ/cm^2 of UV died within a few hours. These dead cells were characterized by swelling of the cytoplasm. Neither nuclear condensation nor fragmentation was observed (Figures 4, B and C, and 5A). A DNA ladder was not demonstrated in this process (Figures 4D and 5D, lane UV 3,000 mJ). On the other hand, mild cellular injuries such as low dosage of UV exposure (10 to 500 mJ), ricin lectin, and cycloheximide showed indirect cell death in which cells died 12 hours to 3 days after the initial exposure. The dead

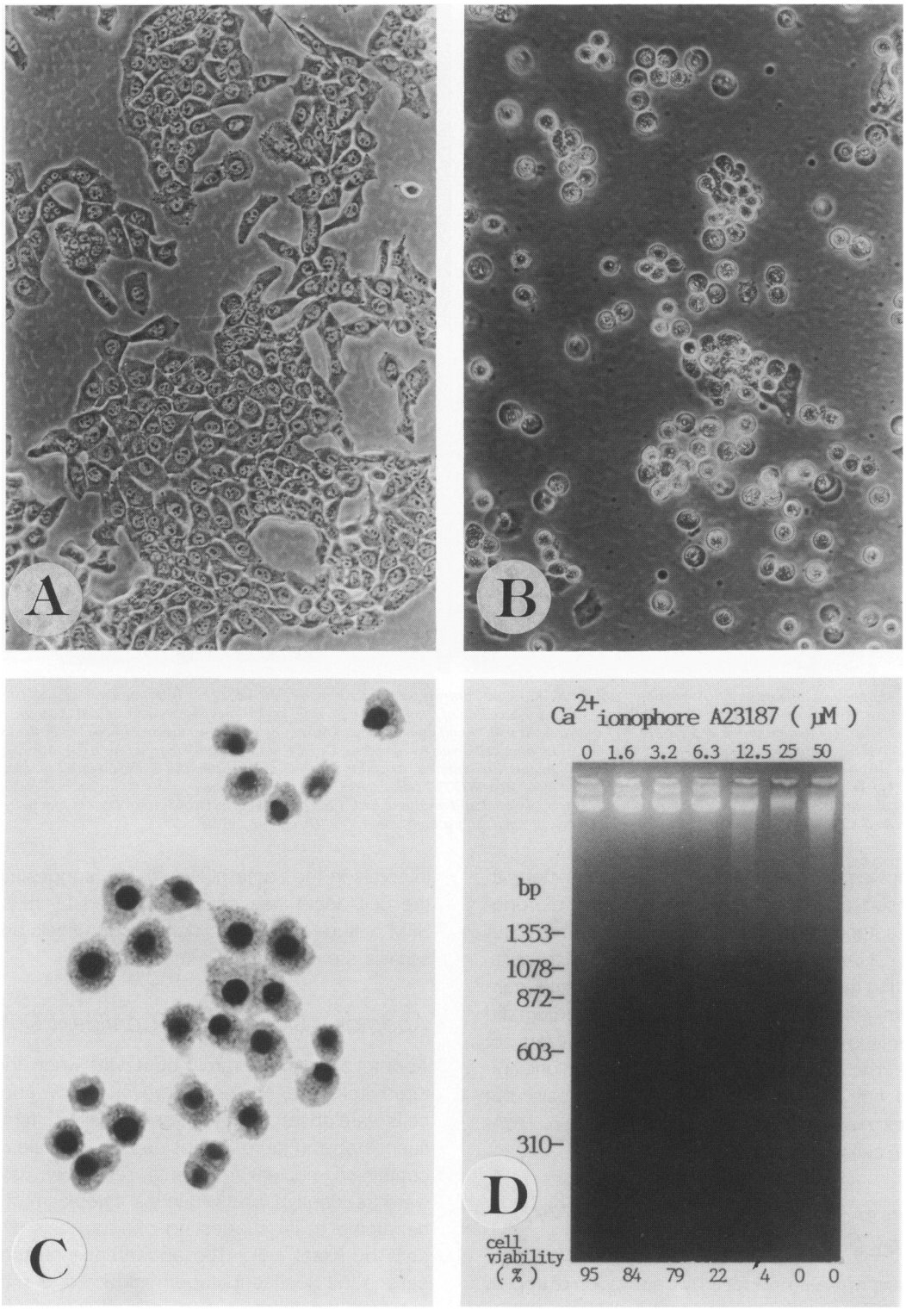

Figure 4. *Cell death in McA-RH7777 cells induced by the Ca^{2+} ionophore A23187.* A: *Phase-contrast photomicrograph of a control culture of McA-RH7777 in medium containing 15% fetal bovine serum (× 140).* B: *Phase-contrast photomicrograph of McA-RH7777 cells treated with 20 μmol/L of Ca^{2+} ionophore A23187 for 1 hour. Dead cells detached from the substratum show swelling of the cells, which suggests cell death by necrosis (× 140).* C: *H&E histology of dead cells observed in* (B) *(× 562).* D: *Electrophoretic analysis of DNAs isolated from McA-RH7777 cells treated with various concentrations of Ca^{2+} ionophore A23187 for 24 hours. Cell viability is expressed as the percentage of cells that excluded trypan blue.*

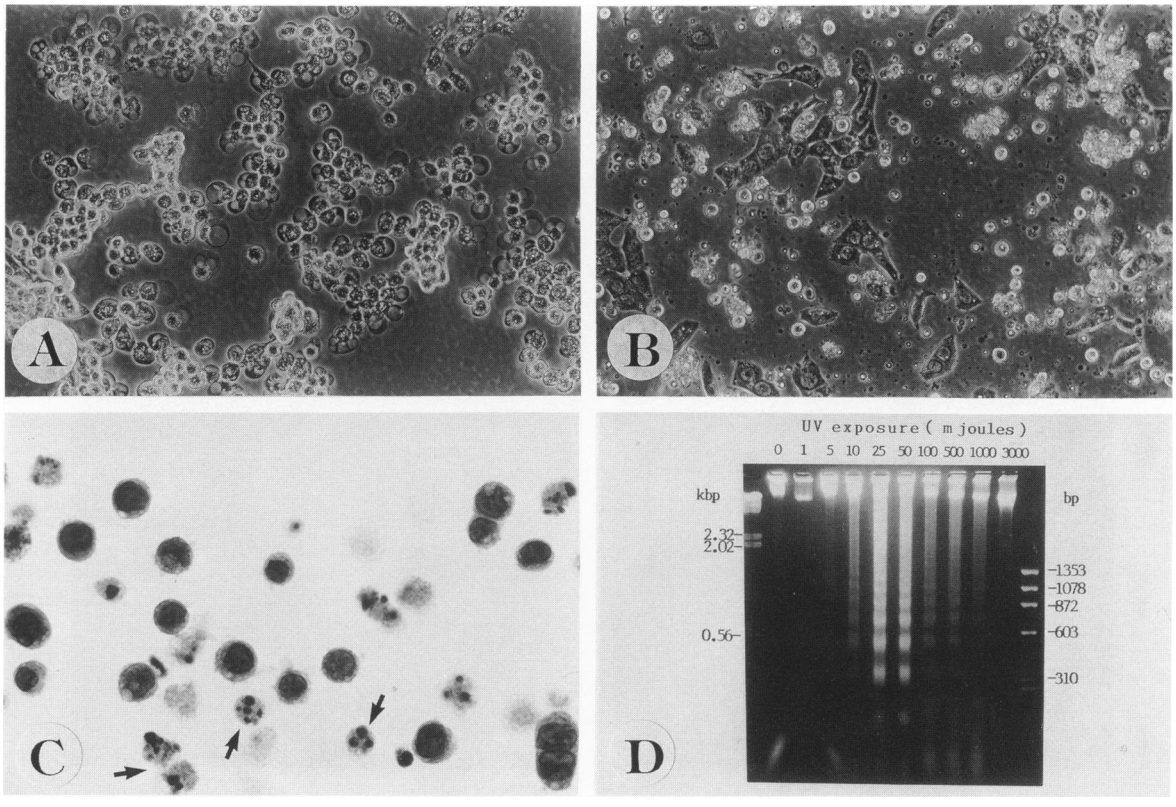

Figure 5. *Morphological features of UV-mediated cell death.* **A:** *Phase-contrast photomicrograph of McA-RH7777 cells treated with 3 × 10³ mJ/cm² of UV exposure. The photo was taken 3 hours after the exposure. Swelling of cytoplasm in floating cells is obvious, indicating cell death by necrosis.* **B:** *Phase-contrast photomicrograph of McA-RH7777 cells treated with 50 mJ/cm² of UV exposure. The photo was taken 24 hours after the exposure. Shrinkage and fragmentation of dead cells are obvious, indicating cell death by apoptosis.* **C:** *H&E stain of cells obtained from* **(B)**. *Many apoptotic cells (some arrowed), which contain condensed nuclear fragments, are observed. Irregular aggregation of chromatin is also observed in many nuclei (H&E, ×431).* **D:** *Electrophoretic analysis of DNAs isolated from McA-RH7777 cells exposed with various dosages of UV (0 to 3 × 10³ mJ/cm²). The cells were collected 24 hours after the exposure, and DNAs were isolated and analyzed with 1.6% agarose gel electrophoresis. DNA ladder was observed in cells treated with 10 to 500 mJ/cm² of UV but not in cells treated with a high dose (3 × 10³ mJ/cm²) of UV.*

cells showed condensed and fragmented nuclei with condensed cytoplasm, which fulfilled the morphological criteria of apoptosis (Figure 5, B and C). A distinct DNA ladder was observed in these cells, supporting the activation of an endonuclease (Figure 5D). Elimination of Ca^{2+} from the culture medium did not affect the appearance of apoptotic cells (data not shown). The protein synthesis inhibitor cycloheximide did not inhibit the process of apoptosis, but cycloheximide itself induced apoptosis in McA-RH7777 cells (data not shown).

Induction of Apoptosis in McA-RH7777 Cells by Serum Deprivation

Apoptosis was also induced by deprivation of trophic factors from culture media. In serum-free culture media, McA-RH7777 cells showed extensive cell death by way of apoptosis 2 to 3 days after serum deprivation (Figure 6). However, cultured cells were not diminished after being in SFM for long periods (more than 2 months with changing medium at 2-day intervals in the same culture flask), suggesting that the cell death was counterbalanced by mitosis in SFM culture and that stimulation of mitosis and apoptosis are concurrent in this system.

Apoptosis Observed in Confluent Culture

Apoptotic cells are rarely observed when the cultured cells are sparse. However, many apoptotic cells were observed in high-cell-density culture (confluent stage) (Figure 7). Eosinophilic globules, often containing nuclear remnants (apoptotic bodies), were frequently found within the cytoplasm of intact hepatoma cells, suggesting phagocytosis of dead cells by intact cells. The appearance of apoptotic cells in the confluent stage might be due in part to nutritional deficiency and accumulation of toxic metabolites in the medium. However, frequent medium changes did not abrogate the appearance of apoptotic cells, and several mitotic cells were frequently observed in the same culture, indicating that stimulation of cell growth and death were concurrent

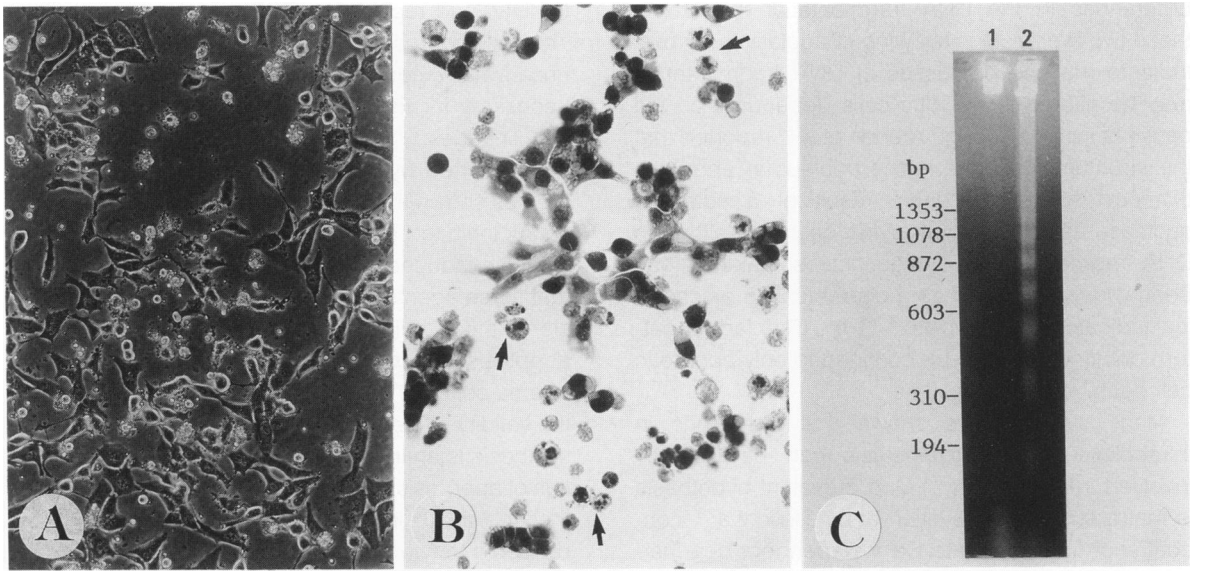

Figure 6. *Demonstration of apoptosis in McA-RH7777 cells cultured in SFM. Phase-contrast photomicrograph* (A) *and H&E stain* (B) *of McA-RH7777 cells cultured in SFM for 3 days. Shrinkage and fragmentation of dead cells are demonstrated in* (A), *and nuclear fragmentation with condensed chromatin is clearly observed in many apoptotic cells (some arrowed) in* (B) (A ×106, B ×212). C: *Electrophoretic analysis of DNAs isolated from control culture in medium containing 15% fetal bovine serum* (lane 1) *and cells cultured in SFM for 3 days* (lane 2).

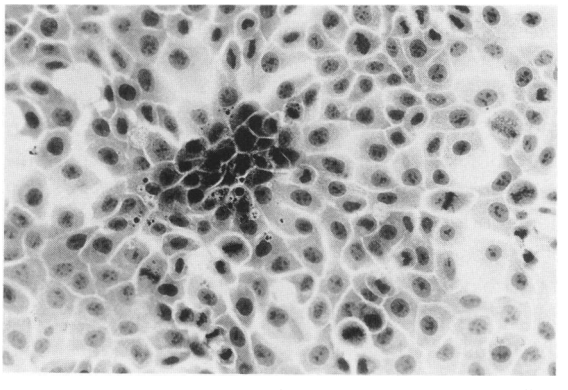

Figure 7. *Histological evidence of apoptosis in McA-RH7777 cells at confluency. Apoptosis is characterized by cell shrinkage and fragmentation. Apoptotic bodies are seen within the cytoplasm of adjacent hepatoma cells. Several mitotic cells are also observed in close vicinity to apoptotic cells (H&E, ×206).*

in the confluent culture of McA-RH7777 cells. This suggests the possibility that the apoptotic process is actively induced to maintain homeostasis of cellular density by counterbalancing mitoses in the expanding tumor population.

Discussion

The focus of molecular oncology is likely to remain on the control of proliferation. However, there is now compelling evidence that the other side of the equation, the rate of cell death, must also be considered.[22] Cell death may occur by necrosis or through the specific process of apoptosis.[2,3] Apoptosis is now widely recognized as a common mechanism for physiological cell deletion and plays a role opposite to that of mitosis in cell population kinetics.[7] Association of tumor suppressor gene p53 with apoptosis has recently been reported, suggesting a close relationship between apoptosis and carcinogenesis.[23]

A number of the steps leading to apoptosis in immature thymocytes and lymphoma cells are well defined.[12–14,24] These include Ca^{2+} influx into the cell and endonuclease activation in the nuclei that produces a characteristic DNA fragmentation (DNA ladder) of multi-nucleosomal length, due to cleavage at the internucleosomal linker region of the chromatin DNA. Apoptotic cells are recognized at the light microscopic level by the characteristic morphology of cell shrinkage with condensed and fragmented nuclei, which are then phagocytosed by macrophages or parenchymal cells.[2,3,7] From these criteria, it is now obvious that the apoptotic process is highly activated in the Morris hepatoma 7777 tissue transplanted into muscle tissue. Distinct DNA ladder patterns were also demonstrated in the liver cells at early stages of ischemic necrosis. Although the steps in the DNA ladder became smaller due to progressive DNA hydrolysis, the ladder pattern was observed against a background of a smear even at 24 hours after ischemia. The DNA ladder was faintly observed as well in the CCl_4-treated liver showing centrilobular coagulative necrosis. The degree of the ladder pattern was, however, very weak in spite of cell death of more than 1/4 of the parenchymal cells.

Differences in the DNA fragmentation pattern in these two liver lesions showing coagulative necrosis indicate that the processes of DNA fragmentation may be altered in necrotic cells depending on the cause of injury. Although Arends et al[14] reported that the structural changes in the nucleus in apoptotic thymocytes are the direct result of a selective nuclease activation within dying cells and although DNA fragmentation into oligonucleosomal units has been widely accepted as a characteristic biochemical indicator for apoptosis[12–14], the DNA ladder pattern does not seem to be confined to only apoptotic cell death in liver cells.

Many studies have shown that alterations in intracellular Ca^{2+} homeostasis can be critically involved in cytotoxicity.[24] One prevalent hypothesis is that a sustained elevation of cytosolic Ca^{2+} concentration can stimulate Ca^{2+}-dependent degenerative enzymes, such as proteases, phospholipases, and nucleases, resulting in irreversible cell damage that leads to cell death.[4–6,17] McConkey et al reported that Ca^{2+} ionophore can induce a DNA ladder in thymocytes and suggested that Ca^{2+} influx is important for the internucleosomal DNA digest.[24] This may not be true of all cell types. Cell death induced by Ca^{2+} ionophore in cultured McA-RH7777 cells was shown to be necrosis, judging from the morphology and DNA fragmentation pattern. Increased level of intracellular Ca^{2+} by Ca^{2+} ionophore does not seem to be sufficient to produce characteristic DNA ladder formation and the morphology of apoptosis in hepatoma cells.

It has recently been suggested that changes in chromatin structure may play a critical role in the induction of DNA cleavage in the early phases of apoptosis.[25] Rat liver nuclei have been known to have endogenous nucleases of the micrococcal nuclease and pancreatic DNAse I type.[15] The former preferentially attacks linker DNA between nucleosomes, giving rise to DNA fragmentation of oligonucleosomal units. The latter type of nuclease produces single-strand cuts in nucleosomal core and internucleosomal DNA to give fragments that are regular multimers of 10 to 11 nucleotides, which may result in a smear pattern of DNA fragmentation in agarose gel electrophoresis. Morris hepatoma 7777 cells have been reported to have low nuclear endonuclease activity.[15] Thus, the activation of internucleosomal DNA cleavage in 7777 cells is a finely regulated active process and is not merely a result of Ca^{2+} influx due to cell damage or death.

The apoptotic process is involved in many physiological and pathological processes in liver tissue, such as normal cell turnover, involution of hyperplastic liver, and regression of preneoplastic cells.[26–30] The cellular process of apoptosis is, however, largely unknown. *De novo* protein synthesis is reported to be necessary for the induction of apoptosis in several cell lineages.[9,13] However, conflicting results are also reported by many investigators.[10,11,31,32] Waring has shown that cycloheximide and the transcriptional inhibitor actinomycin D have no effect on gliotoxin-induced apoptosis of macrophages and that these agents are capable of enhancing apoptosis of these cells.[31] Cycloheximide did not inhibit apoptosis induced by hyperthermia in a Burkitt's lymphoma cell line.[11] All these recent findings question the validity of the widely held view that active protein synthesis is an invariable prerequisite for the execution of apoptosis. In this paper, we demonstrated that the cell death in cultured hepatoma cells can be divided into two major pathways, direct and indirect cell death. Direct cell death, which is inducible by severe cellular damage, demonstrated features of necrosis. On the other hand, indirect cell death, which is inducible by mild cellular injury or elimination of trophic factors, showed the characteristic features of apoptosis. Similar dose-dependent induction of apoptosis by a range of injurious agents were also demonstrated in several hematopoietic cell lines.[10] Lennon et al reported that cells suffering minor injury have the capacity to activate an internally programmed death mechanism, whereas cell death induced by a greater injury may take the form of necrosis.[10] The characteristic DNA ladder and morphological signs of apoptosis were reproducibly demonstrated in McA-RH7777 cells by mild doses of UV exposure. This *in vitro* experimental model seems to be useful in the elucidation of the process of apoptosis in hepatoma cells.

In the earliest study of apoptosis, Kerr demonstrated the induction of apoptosis in normal liver cells by deprivation of the portal blood supply.[33] The SFM that was used in our experiment has been shown to retain liver function and cellular activity for long periods of time in many established hepatoma cell lines.[19] Therefore, it is interesting that the McA-RH7777 cells can proliferate, but that many cells die by way of apoptosis at the same time in SFM. This suggests that McA-RH7777 cells are highly susceptible to apoptosis in the absence of serum growth factors and that cell mitosis and apoptosis are finely regulated in this experimental model.

Even in the presence of serum, many apoptotic cells were demonstrated when the cell density became high (confluent stage). It might be possible that nutritional deficiency and accumulation of toxic metabolites are involved in the process of apoptotic

cell death in confluent culture. However, frequent medium change could not inhibit the appearance of apoptotic cells, and mitotic cells were frequently observed in the same culture, suggesting that the stimulation of mitosis and apoptosis are concurrent in confluent cultures of McA-RH7777 cells. Cell and tissue growth is regulated through a complex interplay of stimulatory and inhibitory signals. It is proposed that proliferation and apoptosis together comprise the regulation of cell growth.[34] The apoptotic process seems to be activated to regulate homeostasis in cell-population kinetics at the confluent stage by counterbalancing mitoses. It is also suggested that a disturbance in oxygen and nutritional supply may be one of the triggering factors in the induction of apoptosis in 7777 tissue.

In conclusion, the cell death observed in transplanted 7777 hepatoma tissue is mainly apoptotic in nature. Based on the results obtained from *in vitro* experiments, it was suggested that tumor apoptosis may represent a residual attempt at autoregulation within the expanding tumor population and/or may result from mild cellular injuries such as hypoxia, nutritional deficiency, or other unknown noxious factor(s).

The induction of apoptosis in cancer cells is one of the major concerns in recent studies in the development of cancer therapy.[8,9,22] A number of studies have explored the attractive idea that tumor cells could be eliminated by artificially triggering cell death through apoptosis.[22,35] Cell-mediated cytotoxicity involves induction of at least some features of apoptosis in the targets.[36] The cell death that is induced by irradiation, hyperthermia, and a range of cytotoxic drugs including many used in cancer therapy has been found to be apoptosis.[8,9,11] Clarification of the biochemical pathways involved in the activation of apoptosis should lead to fundamental advances in the treatment of cancer. Therefore, this cell line seems to be useful as an *in vivo* and *in vitro* experimental model for the elucidation of apoptosis in hepatoma cells.

Acknowledgment

We wish to acknowledge Dr. Roy Korson for critical review of this manuscript.

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